Exhibit 2

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Cyclic Di-GMP Stimulates Protective Innate Immunity in Bacterial Pneumonia▽

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Innate immunity is the primary mechanism by which extracellular bacterial pathogens are effectively cleared from the lung. We have previously shown that cyclic di-GMP (c-di-GMP [c-diguanylate]) is a novel small molecule immunomodulator and immunostimulatory agent that triggers protective host innate immune responses. Using a murine model of bacterial pneumonia, we show that local intranasal (i.n.) or systemic subcutaneous (s.c.) administration of c-di-GMP prior to intratracheal (i.t.) challenge with *Klebsiella pneumoniae* stimulates protective immunity against infection. Specifically, i.n. or s.c. administration of c-di-GMP 48 and 24 h prior to i.t. *K. pneumoniae* challenge resulted in significantly increased survival. Pretreatment with c-di-GMP resulted in a 5-fold reduction in bacterial CFU in the lung ($P < 0.05$) and an impressive >1,000-fold decrease in CFU in the blood ($P < 0.01$). c-di-GMP administration stimulated a robust innate response to bacterial challenge, characterized by enhanced accumulation of neutrophils and αβ T cells, as well as activated NK and αβ T lymphocytes, which was associated with earlier and more vigorous expression of chemokines and type I cytokines. Moreover, lung macrophages recovered from *Klebsiella*-infected mice pretreated with c-di-GMP expressed greater quantities of inducible nitric oxide synthase and nitric oxide ex vivo than did macrophages isolated from infected mice pretreated with the control, c-GMP. These findings demonstrate that c-di-GMP delivered in either a compartmentalized or systemic fashion stimulates protective innate immunity in the lung and protects mice against bacterial invasion. We propose that the cyclic dinucleotide c-di-GMP may be used clinically as an effective immunomodulator, immune enhancer, and vaccine adjuvant to protect against respiratory infection and pneumonia in humans and animals.

Klebsiella pneumoniae is an encapsulated, highly virulent gram-negative bacterium that is a leading cause of both community-acquired and nosocomial pneumonia. A frequent complication of pulmonary infection due to *K. pneumoniae* is the propensity of this organism to spread from the lung into the bloodstream, resulting in widespread systemic dissemination and death. Innate immunity is the principal pathway for elimination of virulent extracellular gram-positive and gram-negative pathogens, including *K. pneumoniae*, from the lung (33). The two main phagocytic cells that constitute pulmonary innate immunity are resident alveolar macrophages and recruited neutrophils (polymorphonuclear leukocytes [PMN]) (28, 45). Both cell types are essential in host defense against bacterial pneumonia, such as that caused by *K. pneumoniae*, as the selective depletion of either cell population results in profound defects in the clearance of bacteria from the alveolar space (4, 47). In addition, local and rapidly recruited lung dendritic cells (DCs) internalize bacteria and promote the expression of type I cytokines by NK cells, T cells, and natural killer T (NKT) cells (2, 9, 10, 24, 25, 42, 19, 26, 27, 29–31). The type 1 cytokines interleukin-12 (IL-12), gamma interferon (IFN-γ), and interferon-inducible protein 10 (IP-10) are required for host defense against both intracellular and extracellular bacterial pathogens (3, 8, 14, 32, 41, 43, 44, 48, 49).

Cyclic di-GMP (c-di-GMP [c-diguanylate]) is an intracellular signaling molecule that was initially identified in the bacterium *Acetobacter xylinum* and shown to regulate the production of cellulose by this microbe (1, 39, 40). More recently, this molecule has been found in multiple bacterial species but not higher eukaryotes (7, 12, 20, 34, 36, 37), and c-di-GMP signaling is believed to be an exclusively bacterial trait. Importantly, c-di-GMP is now recognized to control many key functions in bacteria, including bacterial survival, adhesion, colonization, and biofilm formation (6, 16, 20, 23, 34, 38).

We have previously demonstrated that exogenous chemically synthesized c-di-GMP significantly reduces in vitro cell-cell interactions and biofilm formation of the gram-positive pathogen *Staphylococcus aureus*, including human methicillin-resistant *S. aureus* strains and animal clinical isolates (23). In vivo, we have shown that the intramammary administration of c-di-GMP inhibits colonization and biofilm formation by *S. aureus* in a murine model of mastitis infection, resulting in the enhanced ability of the host to clear the pathogen (5). While there is ample evidence to indicate that c-di-GMP plays a key role in regulating virulence properties of bacteria, there are also emerging data to indicate that this bacterial product might also modulate host cellular responses. Consistent with this notion, we have recently shown that c-di-GMP inhibits basal

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and growth factor-induced proliferation of human colon carcinoma cells (21).

Moreover, we were the first to show that c-di-GMP is an immunomodulatory (immunostimulatory) molecule and has potent immunoprophylactic properties and vaccine adjuvant effects on antibody production, as the intramuscular vaccination of mice with c-di-GMP coinjected with *S. aureus* clumping factor A (ClfA) antigen resulted in significantly higher serum antigen-specific antibody responses than antigen alone (22). The intraperitoneal injection of mice with c-di-GMP promoted monocyte and granulocyte recruitment. Human immature DCs cultured in the presence of c-di-GMP showed increased expression of costimulatory molecules CD80/CD86 and maturation marker CD83, increased major histocompatibility complex class II, and cytokines and chemokines, such as IL-12, IFN-γ, IL-8, monocyte chemoattractant protein 1, IP-10, and RANTES, and altered expression of chemokine receptors, including CCR1, CCR7, and CXCR4. Furthermore, c-di-GMP-matured DCs demonstrated enhanced T-cell stimulatory activity. The immune effects of c-di-GMP were associated with activation of p38 mitogen-activated protein kinase in human DCs and extracellular signal-regulated kinase phosphorylation in human macrophages (22).

To further investigate the immunostimulatory role of c-di-GMP on the innate immune responses of the host in response to, and to protect against, respiratory bacterial invasion, we used an established mouse model of virulent gram-negative bacterial pneumonia. We provide additional direct evidence that c-di-GMP is an immunostimulator and acts as a danger signal to exploit the host immune system and stimulate protective innate antibacterial immunity, as the intranasal (i.n.) administration of synthetic c-di-GMP results in improved lung bacterial clearance and prevention of the systemic sequelae of pneumonia caused by *K. pneumoniae*.

MATERIALS AND METHODS

Animals. Female specific-pathogen-free 6- to 8-week-old BALB/c mice (average weight, approximately 20 g) were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were housed under specific-pathogen-free conditions within the University of Michigan animal care facility until the day of sacrifice.

Bacterial preparation and intratracheal (i.t.) inoculation. The virulent *K. pneumoniae* strain 43816 serotype 2 (ATTC, Manassas, VA) was used in our studies (4, 32, 48). *K. pneumoniae* was grown overnight in tryptic soy broth (Difco, Detroit, MI) at 37°C. The concentration of bacteria in broth was determined by measuring the absorbance at 600 nm and then plotting the optical density on a standard curve generated with known CFU values. The bacterial culture was then diluted to the desired concentration.

Mice were anesthetized with an intraperitoneal mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). For i.n. administration, c-di-GMP, the nucleotide control c-GMP, or vehicle (phosphate-buffered saline [PBS]) was administered i.n. in a 10-μl volume. For i.t. administration of bacteria, the trachea was exposed, and 30 μl of inoculum was administered via a sterile 26-gauge needle. The skin incision was closed using surgical staples.

c-di-GMP. The c-di-GMP used in these studies was chemically synthesized and prepared as described previously (15, 17, 18). The purity of the batch of c-di-GMP used was >98% and was confirmed by high-performance liquid chromatography, ^{31}P-nuclear magnetic resonance, and electrospray ionization –time-of-flight mass spectrometry analyses (data not shown). Control c-GMP was purchased from Sigma (St. Louis, MO). c-di-GMP or control c-GMP was reconstituted at the appropriate concentration in 30 μl of sterile saline or water. Control groups in the experiments received either vehicle (PBS) alone or control c-GMP (Sigma). Effects of control c-GMP were similar to vehicle alone, and for that reason c-GMP was used as the control for most of the experiments performed. All GMP preparations (including c-di-GMP and c-GMP) were free of endotoxin contamination as determined by a *Limulus* assay.

Whole-lung homogenization for *K. pneumoniae* CFU determination. At designated time points, the mice were euthanized by CO_2 inhalation. Prior to lung removal, the pulmonary vasculature was perfused by infusing 1 ml of PBS containing 5 mM EDTA into the right ventricle. Whole lungs were removed, taking care to dissect away lymph nodes. The lungs were then homogenized in 1 ml of PBS with protease inhibitor (Boehringer Mannheim, Indianapolis, IN). Homogenates were then serially diluted 1:5 in PBS and plated on blood agar to determine lung CFU. The remaining homogenates were sonicated and then centrifuged at 1,400 × *g* for 15 min. Supernatants were collected, passed through a 0.45-μm-pore-size filter, and then stored at −20°C for assessment of cytokine levels.

Peripheral blood CFU. Blood was collected in a heparinized syringe from the right ventricle at the designated time points, serially diluted 1:2 with PBS, and plated on blood agar to determine blood CFU.

Total lung leukocyte isolation and cytospins. Total lung leukocytes were isolated as previously described (9, 14). Briefly, lung tissue was minced to a fine slurry in 15 ml of digestion buffer (RPMI, 5% fetal calf serum, collagenase at 1 mg/ml [Boehringer-Mannheim, Chicago, IL], and DNase at 30 μg/ml [Sigma, St. Louis, MO]). Lung slurries were enzymatically digested for 30 min at 37°C. Undigested fragments were further dispersed by drawing the solution up and down through the bore of a 10-ml syringe. The total lung cell suspension was pelleted, resuspended, and spun through a 20% Percoll gradient to enrich for leukocytes for flow analysis. Cell counts and viability were determined on a hemacytometer using trypan blue exclusion. Cytocentrifugation slides (Cytospin 2; Shandon Inc., Pittsburgh, PA) were prepared from lung digest leukocyte suspensions and stained with Diff-Quik (Dade Behring, Newark, DE) for cell differential determinations.

Measurement of NO. Lung macrophages were isolated from lung digest cells by 20% Percoll gradient enrichment and adherence purification at a concentration of 5×10^5 cells/well. Cells were washed three times and then incubated for 18 h in RPMI plus penicillin-streptomycin. The production of nitric oxide (NO) was determined by measuring the accumulation of nitrite, the stable metabolite of NO, in culture medium based on the Griess reaction as previously described (46). A standard nitrite curve was generated in the same fashion using $NaNO_2$.

Real-time quantitative reverse transcription-PCR. Whole lung was harvested at the designated time points, immediately snap-frozen in liquid nitrogen, and then stored at −70°C for RNA extraction. Total cellular RNA was isolated from frozen lungs as described previously. Measurement of gene expression was performed utilizing the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as previously described (47). Briefly, primers and probes were designed using the Shortcut to Primer Express software (Applied Biosystems). The primers, placed in different exons, were tested not to amplify genomic DNA. Primers and probe nucleotide sequences were as follows: for mouse IP-10 (mIP-10), forward primer, 5′-CCA GTG AGA ATG AGG GCC ATA-3′, reverse primer, 5′-CTC AAC ACG TGG GCA GGA T-3′, and TaqMan probe, 5′–6-carboxyfluorescein–TTT GGG CAT CAT CTT CCT GGA–tetramethylrhodamine–3′; for mouse tumor necrosis factor alpha (mTNF-α), forward, 5′-CAG CCG ATG GGT TGT ACC TT-3′, reverse, 5′-TGT GGG TGA GGA GCA CGT AGT-3′, and probe, 5′-TCC CAG GTT CTC TTC AAG GGA CAA GGC-3′; for mouse macrophage-inflammatory protein (mMIP-2), forward, 5′-GAA CAT CCA GAG CTT GAG TGT GA-3′, reverse, 5′-CCT TGA GAG TGG CTA TGA CTT CTG T-3′, and probe, 5′-CCC CCA GGA CCC CAC TGC G-3′; for mIL-12 p40, forward, 5′-AGA CCC TGC CCA TTG AAC TG-3′, reverse, 5′-GAA GCT GGT GCT GTA GTT CTC ATA TT-3′, and probe, 5′-CGT TGG AAG CAC GGC AGC AGA A-3′; for mouse inducible NO synthase (miNOS), forward, 5′-CCC TCC TGA TCT TGT GTT GGA-3′, reverse, 5′-CAA CCC GAG CTC CTG GAA-3′, and probe, 5′-TGA CCA TGG AGC ATC CCA AGT ACG AGT-3′; for mβ-actin, forward, 5′-CCG-TGA-AAA-GAT-GAC-CCA-GAT-C-3′, reverse, 5′-CAC-AGC-CTG-GAT-GGC-TAC-GT-3′, and probe, 5′-TTT-GAG-ACC-TTC-AAC-ACC-CCA-GCC-A-3′. Specific thermal cycling parameters used with the TaqMan one-step reverse transcription-PCR Master Mix reagents kit included 30 min at 48°C, 10 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Relative quantitation of cytokine mRNA levels was plotted as the change compared to that with untreated control lung. All experiments were performed in duplicate.

Multiparameter flow cytometric analysis. Total lung leukocytes were isolated as described previously (9, 48). Using fluorescein isothiocyanate- or phycoerythrin-labeled antibodies (BD PharMingen, San Diego, CA), isolated leukocytes were then stained with the following: anti-F4/80 (macrophage), anti-CD4, anti-CD8, anti-αβ-Tcr (αβ T-cell marker), anti-γδ-Tcr (γδ T-cell marker), anti-

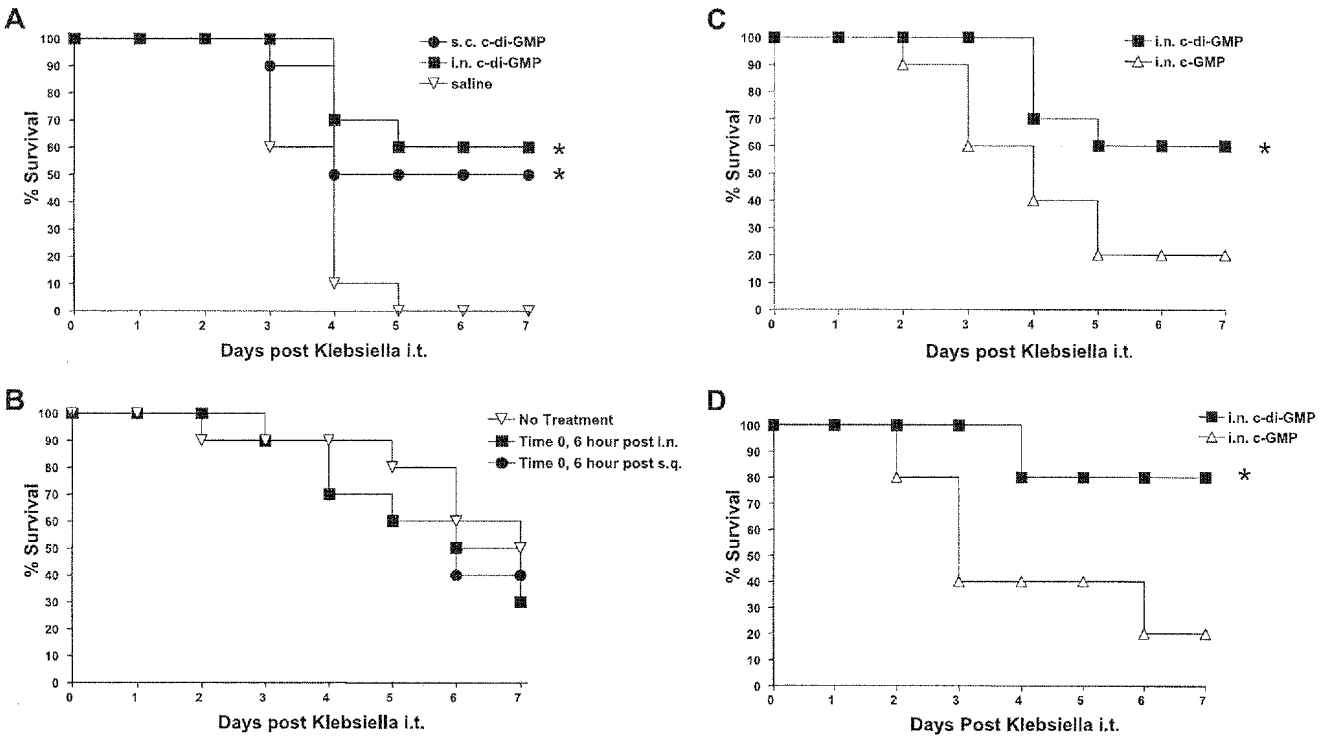

FIG. 1. Survival following treatment with either i.n. or s.c. c-di-GMP in *Klebsiella*-infected animals. A. Animals were administered i.n. or s.c. c-di-GMP or vehicle (saline) 48 and 24 h prior to i.t. *K. pneumoniae* (5×10^3) challenge. B. Animals were administered i.n. or s.c. c-di-GMP or vehicle concomitant with and 6 h after i.t. *K. pneumoniae* (1×10^3 to 2×10^3) challenge. C. Animals were administered i.n. c-di-GMP or control cGMP 48 and 24 h prior to challenge with a lower dose of *K. pneumoniae* (1×10^3 to 2×10^3). D. Animals were administered i.n. c-di-GMP or control c-GMP 24 h prior to challenge with *K. pneumoniae* (3×10^3 to 4×10^3). Survival in all experiments was assessed through day 7. *, $P \leq 0.05$ by two-tailed log rank test compared to control vehicle- or c-GMP-treated *Klebsiella*-infected mice. For panels A to C, data are for 10 to 11 animals per group, with composite results from two separate experiments; for panel D, there were five animals per group. The percent survival is shown on the x axis.

DX5 (NK cell marker), and anti-CD69 antibodies. In addition, cells were stained with anti-CD45-Tricolor (Caltag Laboratories, South San Francisco, CA) to distinguish leukocytes from nonleukocytes. Cells were collected on a FACSCalibur cytometer (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson). NK cells and T-cell subsets were analyzed after gating on $CD45^+$ lymphocyte-sized cells and then examining for FL-1 and FL-2 fluorescence expression. Neutrophils were identified in cytospin preparations by morphological criteria.

Statistical analysis. Survival curves were compared using the log rank test. For other data, statistical significance was determined using the unpaired t test or analysis of variance for multiple comparisons as appropriate. All calculations were performed using the Prism 3.0 software program for Windows (GraphPad Software, Inc., San Diego, CA).

RESULTS

Effect of c-di-GMP administration on survival in murine *Klebsiella* pneumonia. To determine the effect of c-di-GMP pretreatment on survival in mice challenged with *K. pneumoniae*, BALB/c mice were treated with c-di-GMP (200 nmol; equivalent to a dose of approximately 2.5 mg/kg) or vehicle i.n. or subcutaneously (s.c.) at both 48 and 24 h prior to the i.t. administration of *K. pneumoniae* (5×10^3 CFU) and then assessed for survival. In previously published mouse studies, it was shown that the maximal benefit of c-di-GMP was observed at an in vivo dose of 200 nmol/animal (5). In the current studies, no control *Klebsiella*-infected animals pretreated with vehicle survived past 5 days post-infectious challenge. In contrast, survival of mice pretreated with c-di-GMP either locally (i.n.) or systemically (s.c.) was significantly increased at both early and late time points following i.t. *Klebsiella* challenge (Fig. 1A) ($P < 0.05$), with the i.n. route of delivery being equally as efficacious as the s.c. route. Importantly, the survival benefits observed required pretreatment, as no increase in survival was observed in animals in which c-di-GMP was administered i.n. or s.c. concomitant with and 6 h post-*K. pneumoniae* administration (Fig. 1B). Improved survival was also observed in mice pretreated (48 and 24 h pre-*Klebsiella*) with c-di-GMP intranasally and then challenged with a lower dose of *K. pneumoniae* (2×10^3 CFU), compared to mice pretreated with control c-GMP (Fig. 1C) ($P < 0.05$). Moreover, we found that a single i.n. administration of c-di-GMP at 24 h prior to *K. pneumoniae* afforded a similar magnitude of protection as that observed with c-di-GMP administration at both 48 and 24 h prior to bacterial challenge (Fig. 1D).

Effect of c-di-GMP pretreatment on *K. pneumoniae* clearance in a murine model of pneumonia. Experiments were performed to determine if the beneficial effect of c-di-GMP was attributable to improved bacterial clearance and decreased dissemination. Because we observed that the survival benefits of c-di-GMP when given by the i.n. route were equivalent to those observed with s.c. administration, subsequent studies were performed using the i.n. route of c-di-GMP delivery. In

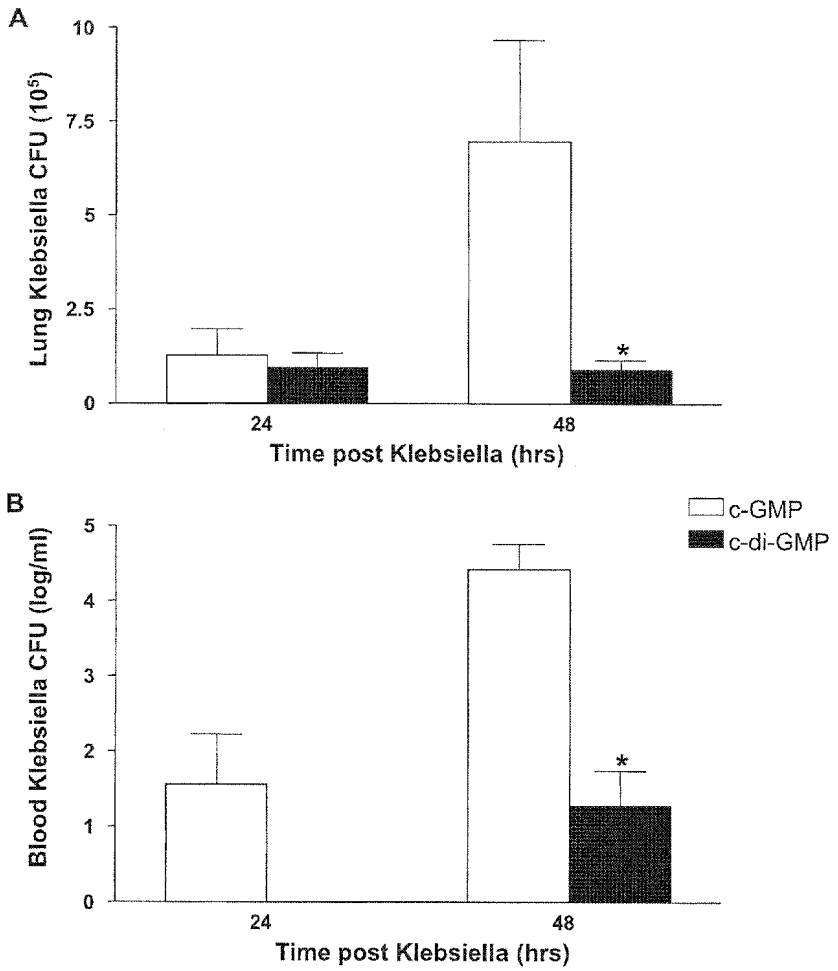

FIG. 2. Bacterial clearance in *Klebsiella*-infected animals pretreated with i.n. c-di-GMP or control c-GMP. Mice were administered i.n. c-di-GMP or control 48 and 24 h prior to i.t. *Klebsiella*, and then *K. pneumoniae* CFU were determined in lung and blood 24 and 48 h later. (A) Lung CFU; (B) blood CFU. Bacterial CFU in lung is shown on an algebraic scale on the *x* axis, whereas blood CFU is shown as the $\log_{10} \pm$ the standard error of the mean on the *x* axis and is composited from two different experiments. *, $P < 0.01$ compared to i.n. control c-GMP (experimental group, $n = 6$ to 9 animals).

these studies, mice were pretreated with c-di-GMP (200 nmol) or control c-GMP i.n. at 48 and 24 h prior to i.t. *K. pneumoniae* (5×10^3 CFU) challenge, and then bacterial burdens in lung and blood were determined at 24 and 48 h post-bacterial challenge. Intranasal pretreatment with c-di-GMP resulted in an approximately fivefold reduction in *K. pneumoniae* CFU in lung at 48 h postinfection ($P < 0.05$) (Fig. 2). More striking was the finding that i.n. pretreatment with c-di-GMP tended to prevent the development of bacteremia at 24 h ($P = 0.07$ compared to control c-GMP-treated mice) and at 48 h resulted in a greater-than-1,000-fold reduction in *K. pneumoniae* CFU in blood, compared to that observed in control infected animals ($P < 0.01$). As an important control, we found that c-di-GMP in concentrations ranging from 2 nM to 200 nM had no direct inhibitory effect on growth of *K. pneumoniae* in culture (data not shown).

Effect of c-di-GMP pretreatment on lung leukocyte influx in murine *Klebsiella* pneumonia. We next determined if pretreatment with c-di-GMP enhanced bacterial clearance in murine *Klebsiella* pneumonia by altering the influx and/or activation of cells required for effective antibacterial host defense. To address this, mice were administered either c-di-GMP or equal concentrations of nucleotide control (c-GMP) i.n. 48 and 24 h prior to i.t. *K. pneumoniae* challenge, and then lungs were harvested 24 and 48 h postinfection and total leukocyte populations were quantitated by lung digestion. As shown in Fig. 3, i.t. administration of *K. pneumoniae* resulted in an increase in the total number of leukocytes, especially neutrophils, in whole lung digest, compared to uninfected controls. No difference in total lung leukocytes or PMN was noted between control c-GMP- and c-di-GMP-treated mice at 24 h post-*K. pneumoniae* administration. However, animals pretreated with c-di-GMP had a significant increase in the total number of lung leukocytes ($P < 0.01$) and PMN ($P < 0.01$) at 48 h compared to animals pretreated with c-GMP control. No changes in the numbers of lung macrophages, plasmacytoid dendritic cells, or myeloid dendritic cells were noted between the two groups at either 24 or 48 h (data not shown).

To determine if c-di-GMP administration altered the influx and/or activation of selected T-cell and NK cell populations,

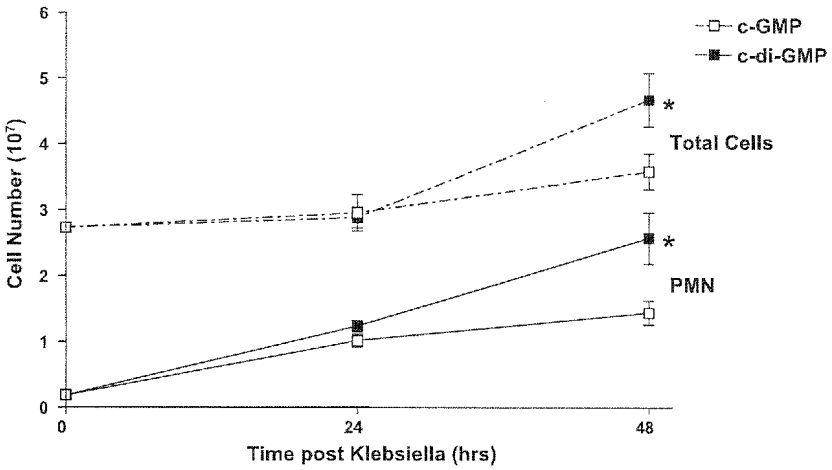

FIG. 3. Lung leukocyte influx post-*K. pneumoniae* administration in animals pretreated with i.n. c-di-GMP or control c-GMP. Total lung leukocytes (dashed line) and PMN (solid line) were quantitated in lung digests 24 and 48 h after *K. pneumoniae* administration. *, $P < 0.05$ compared to *Klebsiella*-infected mice pretreated with control (c-GMP) i.n. $n = 3$ for the uninfected group, and $n = 6$ for control c-GMP- and c-di-GMP-treated groups.

animals were pretreated with c-di-GMP or vehicle control 48 and 24 h prior to *K. pneumoniae* challenge, and then the presence of specific T and NK cell populations was determined by flow cytometry 48 h post-bacterial challenge. Cellular activation was determined by expression of the activation marker CD69. *Klebsiella* administration in control c-GMP-pretreated animals resulted in an increase in the total number of NK cells and NK cells expressing CD69. Pretreatment with c-di-GMP of infected mice resulted in a trend toward further increased numbers of NK cells and a significant increase in the accumulation of NK cells expressing CD69 (Table 1). Moreover, we observed an increase in the number of αβ T cells in lungs of infected mice pretreated with c-di-GMP compared to controls, as well as a significantly greater number of activated αβ T cells (as indicated by CD69 expression) in animals pretreated with c-di-GMP. No differences in the number of total or activated γδ T cells (data not shown) or NKT cells were observed between the two groups.

Effect of c-di-GMP administration on the expression of chemotactic and activating cytokines in uninfected and *Klebsiella*-infected mice. The previous experiments indicated that the i.n. administration of c-di-GMP resulted in a significant increase in lung neutrophils, as well as an accumulation and/or activation of NK and αβ T-cell populations in mice infected with *K. pneumoniae*. To define the mechanism of enhanced neutrophil recruitment and selected NK and T-cell accumulation/activation, we assessed the time-dependent expression of TNF-α, IL-17, the neutrophil chemotactic cytokine MIP-2, and the type I cytokines IL-12, IFN-γ, and IP-10 in the lungs of animals pretreated with c-di-GMP or control c-GMP. Pretreatment of mice with c-di-GMP did not significantly alter the induction of TNF-α or IL-17 in response to i.t. bacterial administration (data not shown). However, we observed a significantly greater early induction of MIP-2 mRNA levels (at 24 h post-*Klebsiella* challenge) compared to that observed in animals pretreated with the c-GMP control (twofold increase over infected controls; $P < 0.05$) (Fig. 4). In addition, pretreatment with c-di-GMP i.n. resulted in induction of the type I cytokines IL-12 p40, IFN-γ, and IP-10 in the lungs at time zero (preinfection). Moreover, IP-10 and IFN-γ mRNA levels were significantly higher in c-di-GMP-treated mice at 24 h post-*K. pneumoniae* administration ($P < 0.05$), whereas IL-12 p40 levels were increased at both 24 and 48 h post-bacterial challenge, compared to infected animals pretreated with control c-GMP. Pretreatment with c-di-GMP also enhanced the production of MIP-2, IL-12, IFN-γ, and IP-10 protein levels at 24 and/or 48 h post-*K. pneumoniae* administration, compared to control-treated animals (Table 2).

Effect of c-di-GMP administration on expression of iNOS and production of nitric oxide by lung macrophages ex vivo. Nitric oxide has previously been shown to be a required component of effective lung innate immunity in gram-negative bac-

TABLE 1. Effect of c-di-GMP on mononuclear cell accumulation and activation

Cell population	No. of leukocytes[a]		
	Uninfected	c-di-GMP + *K. pneumoniae*	c-GMP + *K. pneumoniae*
$DX5^+$ (NK)	$(1.3 \pm 0.3) \times 10^6$	$(3.8 \pm 0.5) \times 10^6$	$(2.5 \pm 0.4) \times 10^6$
$DX5^+$ $CD69^+$	$(0.8 \pm 0.1) \times 10^5$	$(6.0 \pm 1.0) \times 10^5$†	$(2.7 \pm 0.3) \times 10^5$
$DX5^+$ αβ γδ TCR^+ (NKT)	$(1.0 \pm 0.1) \times 10^5$	$(4.6 \pm 0.5) \times 10^5$	$(4.4 \pm 0.7) \times 10^5$
αβ TCR^+	$(2.6 \pm 0.1) \times 10^6$	$(5.5 \pm 0.6) \times 10^6$	$(3.4 \pm 0.3) \times 10^6$
αβ TCR^+ $CD69^+$	$(0.3 \pm 0.05) \times 10^5$	$(3.7 \pm 0.5) \times 10^5$*	$(2.7 \pm 0.3) \times 10^5$

[a] Leukocytes were quantitated in lung digests 48 h after *K. pneumoniae* administration. *, $P < 0.05$; †, $P < 0.01$ compared to *Klebsiella*-infected mice pretreated with control (c-GMP) i.n. $n = 3$ for uninfected animals; $n = 6$ for the c-GMP control- and c-di-GMP-treated groups.

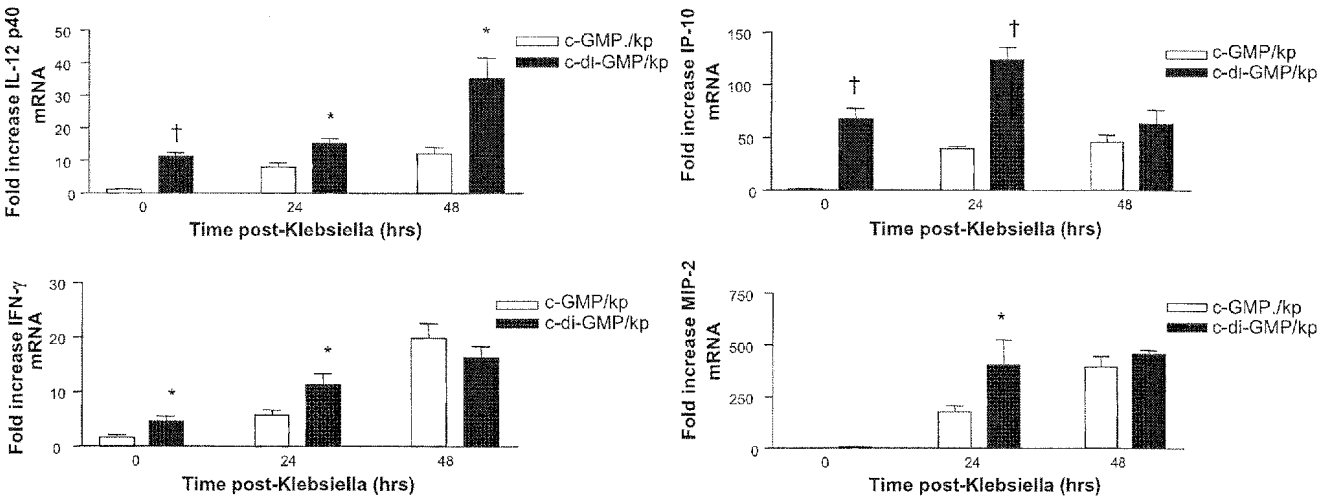

FIG. 4. Cytokine mRNA levels in whole lung homogenates following i.n c-di-GMP or control treatment and i.t. *Klebsiella pneumoniae* challenge. Mice were administered i.n. c-di-GMP or control c-GMP 48 and 24 h prior to i.t. *K. pneumoniae*, and then cytokine mRNA levels in lung homogenates were determined at 0, 24, and 48 h post-*K. pneumoniae* (Kleb) challenge by quantitative PCR. Values shown represent the mean fold increase over uninfected control mice (*x* axis). *, $P < 0.05$; †, $P < 0.01$ compared to control-treated animals (four to five animals per group).

terial pneumonia (46). To determine if the enhanced bacterial clearance and improved survival observed in c-di-GMP-pretreated mice were attributable, in part, to augmented NO synthesis by pulmonary macrophages, we assessed the time-dependent expression of iNOS mRNA and spontaneous production of NO from ex vivo cultured lung macrophages isolated from control or c-di-GMP-pretreated mice at 24 and/or 48 h post-i.t. *K. pneumoniae* administration. In these experiments, macrophages were isolated from $CD45^+$ lung digest cells after Percoll gradient centrifugation and adherence purification. As shown in Fig. 5A, spontaneous iNOS mRNA expression by lung macrophages peaked at 48 h post-bacterial administration and was substantially greater in macrophages isolated from c-di-GMP-pretreated mice than in control c-GMP-pretreated animals ($P < 0.05$). Furthermore, in Fig. 5B, lung macrophages from c-di-GMP-pretreated mice isolated 48 h post-bacterial challenge produced more NO spontaneously ex vivo than did macrophages from control infected animals ($P < 0.05$).

TABLE 2. Lung homogenate cytokine levels at 24 and 48 h post-*K. pneumoniae* infection[a]

Cytokine	Cytokine level (ng/lung) at time post-*K. pneumoniae* infection				
	Uninfected	24 h		48 h	
		c-di-GMP	c-GMP	c-di-GMP	c-GMP
MIP-2	0.34 ± 0.12	2.02 ± 0.36	1.82 ± 0.44	10.05 ± 2.30*	5.34 ± 0.91
IP-10	0.47 ± 0.10	2.51 ± 0.35*	1.17 ± 0.32	4.18 ± 0.76	3.51 ± 0.71
IL-12	0.17 ± 0.06	0.95 ± 0.20*	0.44 ± 0.04	1.41 ± 0.23*	0.082 ± 0.11
IFN-γ	0.14 ± 0.08	1.59 ± 0.26*	0.80 ± 0.09	1.00 ± 0.30	0.65 ± 0.22

[a] Animals were pretreated with c-di-GMP or control c-GMP at 48 and 24 h before *K. pneumoniae* administration. *, $P < 0.05$ compared to control infected mice.

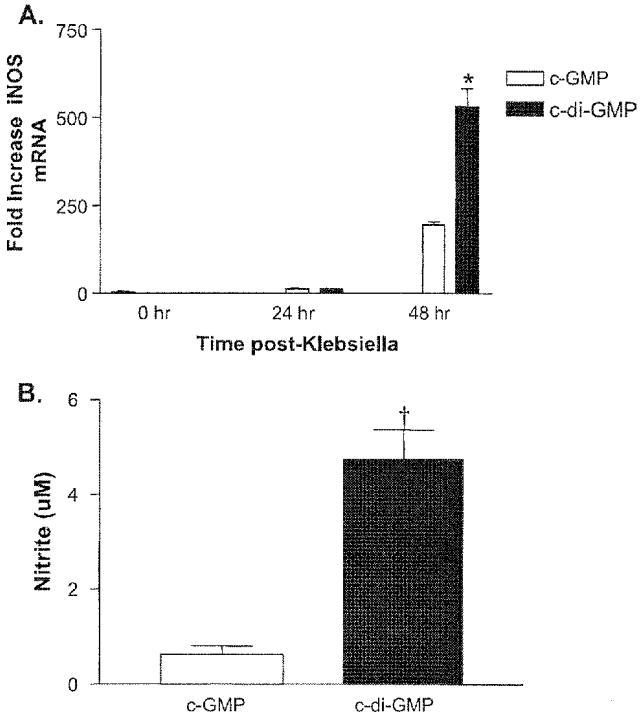

FIG. 5. Spontaneous iNOS mRNA expression and nitric oxide production by lung macrophages post-i.t. *K. pneumoniae*. (A) Constitutive iNOS mRNA expression by lung digest macrophages isolated at 24 and 48 h post-i.t. *K. pneumoniae* administration and expressed as fold increase over lung macrophages from uninfected mice. (B) Spontaneous nitrite production (as a measure of NO expression) after 18 h in culture from lung macrophages isolated from mice 48 h post-i.t. bacterial administration. Lung macrophages were pooled from four mice at each time point and condition. *, $P < 0.05$; †, $P < 0.01$ compared to the control c-GMP-treated group.

DISCUSSION

In this study, we report that pretreatment of mice with either i.n. or systemic (s.c.) c-di-GMP, but not control nucleotide (c-GMP) or vehicle (saline), can induce a significant protective immune response against i.t. challenge with a virulent strain of *K. pneumoniae* in a mouse model of bacterial pneumonia. Our findings with *K. pneumoniae* (a gram-negative pathogen) are consistent with a previous study showing improved bacterial clearance of *S. aureus* (a gram-positive pathogen) in a mouse infection model after pretreatment with the small-molecule cyclic dinucleotide c-di-GMP (5), as well as with our recent in vivo and in vitro studies showing that c-di-GMP is an immunomodulator and immunostimulatory molecule (22).

In bacterial pneumonia, clearance of pathogens, especially virulent gram-negative pathogens, is primarily dependent upon a vigorous innate immune response (4, 28, 45, 46). This study demonstrates that c-di-GMP administration enhances several key aspects of the cytokine-mediated innate immunity in the lung. In particular, c-di-GMP primes the host for enhanced early expression of MIP-2, a potent neutrophil-active chemokine that contributes to the recruitment of neutrophils in gram-negative pneumonia. Moreover, we observed that c-di-GMP given i.n. directly stimulated the expression of type I cytokines/chemokines IL-12, IFN-γ, and IP-10, and the expression of these cytokines was further enhanced during lung bacterial infection. This is of particular clinical relevance, as these cytokines are necessary for effective clearance of *K. pneumoniae* and many other important bacterial pathogens from the lung (14, 48, 49). Our previous study (22) demonstrated that TNF-α was short lived, peaked 3 h after c-di-GMP treatment, and returned to near-basal levels after 24 h. In the current study, we found that TNF-α was not significantly affected at the time points examined. This is important, since excessive and unchecked release of TNF-α can lead to tissue damage and sepsis. Collectively, the specific and controlled presence of cytokines indicates that c-di-GMP functions as an effective immunostimulatory molecule that skews the immune system towards a beneficial type I phenotype cytokine response, an effect which is clinically advantageous in host defenses against both intracellular and extracellular bacterial pathogens.

The cellular components of c-di-GMP-stimulated immunity have not been clearly defined in our model, but several candidate cell populations are likely involved. We observed a more robust influx of neutrophils in c-di-GMP-pretreated animals post-bacterial challenge, particularly at the 48-h time point. Neutrophils represent an important phagocytic cell in the clearance of bacterial pathogens from the lung (28, 47). The c-di-GMP-induced upregulation of MIP-2 may contribute to enhanced neutrophil trafficking. Moreover NK, NKT, and αβ T cells are populations that play an important role in innate immunity. For instance, activated NK cells are considered to be the primary source of IFN-γ in the lung early in the course of bacterial infection (8, 10). In addition, increased numbers of αβ T cells, as well as enhanced activation of NK and αβ T cells, was found in the lungs of *Klebsiella*-infected animals pretreated with c-di-GMP, compared to animals pretreated with the control. The accumulation and/or activation of these cell populations in the lungs of c-di-GMP-pretreated animals may be partially attributable to the enhanced expression of IP-10, which is a chemoattractant for these cells in vivo and in vitro (11, 19, 35). Thus, the recruitment and/or activation of several key immune cell populations likely contributes to improved bacterial clearance and outcome in animals pretreated with c-di-GMP.

Additionally, lung macrophages represent an important component of antibacterial innate immunity in pneumonia. The internalization and intracellular killing of bacteria by alveolar and interstitial lung macrophages represent the first line of defense against bacteria that have reached the distal airspaces (28, 45). Macrophage microbicidal activity is partially dependent on the generation of NO, which is required for effective clearance of bacteria in murine *Klebsiella* pneumonia (46). We observed enhanced expression of iNOS and NO by pulmonary macrophages recovered from infected mice pretreated with c-di-GMP. Type I cytokines, including IFN-γ, are inducers of iNOS, and we found increased early expression of IFN-γ in c-di-GMP-pretreated animals.

The promotion of enhanced type I immunity in response to c-di-GMP administration supports the distinct possibility that c-di-GMP directly stimulates DC-mediated responses. In fact, we have recently shown that c-di-GMP induces DC cytokine and chemokine production and increases the cell surface expression of maturation markers, including CD80, CD86, CCR7, and major histocompatibility complex class II (22). In addition, that study also showed that stimulation of human DCs by c-di-GMP was associated with activation of p38 mitogen-activated protein kinase. The finding of enhanced IL-12 p40 expression in response to c-di-GMP is consistent with DC activation in the lung, although cells other than DCs may also contribute to enhanced IL-12 expression, including lung macrophages. Notably, the i.n. administration of c-di-GMP did not alter trafficking of myeloid or plasmacytoid DCs to the lung in response to bacterial challenge, nor did it change the expression of costimulatory molecules (CD40, CD80, or CD86) by lung myeloid DCs (data not shown).

In this study, we demonstrated that chemically synthesized c-di-GMP, a bacterially derived intracellular signaling molecule, can prime or directly stimulate multiple beneficial aspects of innate immunity. We propose that c-di-GMP treatment may play a potentially beneficial role in immunoprophylaxis as a general immune enhancer by activating innate host defenses in humans or animals with respiratory infections and pneumonia or in those who are at high risk for the development respiratory infections and pneumonia.

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D.K.R. Karaolis has three related patents: a method for attenuating virulence of microbial pathogens and for inhibiting microbial biofilm formation (PCT/US04/23498); a method for stimulating the immune, inflammatory, or neuroprotective response (U.S. 11/079, 886; PCT/

US05/08447); and a method for inhibiting cancer cell proliferation or increasing cancer cell apoptosis (U.S. 11/079,779; PCT/US05/08448).

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